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Chemistry

Carbon monoxide binding to iron porphyrins

(picket fence porphyrins/hemoproteins/distal residues/partition coefficient/solvation)

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ABSTRACT **The carbon monoxide affinities of iron complexes of *meso*-tetra ($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrin (the "picket fence" porphyrin) and of a "picket fence" porphyrin derivative with an appended axial base have been measured in solution and compared with the CO affinities of various hemoproteins. The model complexes bind CO with much greater affinity than normal hemoproteins; the role of the steric bulk of distal residues in lowering the CO affinities of the hemoproteins is discussed. The significance of this lowered CO affinity is described with regard to endogenous CO. A discussion of mutant hemoglobins lacking distal residues that sterically inhibit the binding of CO is presented. The use of pressure units versus concentration units in equilibrium expressions is analyzed.**

The oxygen affinities of myoglobin (Mb) and both the low-affinity ("T") and high-affinity ("R") states of hemoglobin (Hb) have been reproduced by model iron(II) porphyrin complexes (1, 2). Simple iron(II) porphyrins irreversibly oxidize when exposed to oxygen at room temperature in solution (3–6); this led to the development of "picket fence" (7) and "tailed picket fence" (unpublished results) porphyrins whose steric bulk inhibits the oxidation process. The equilibria of interest in the study of such systems are:

$$\mathrm{FeP} + \mathrm{B} \overset{K_1}{\rightleftharpoons} \mathrm{FePB}$$

$$\mathrm{FePB} + \mathrm{B} \overset{K_2}{\rightleftharpoons} \mathrm{FePB_2}$$

$$\mathrm{FePB} + \mathrm{O_2} \overset{K_{O_2}}{\rightleftharpoons} \mathrm{FePB(O_2)},$$

where P represents the porphyrinato ligand and B represents an axial base. For sterically hindered bases, such as 1,2-dimethylimidazole (Me_2Im), K_2 is much less than K_1 (8–10) and direct solution measurements of K_{O_2} are possible. For unhindered bases, such as 1-methylimidazole (*N*-MeIm), however, K_2 is greater than K_1 (10), precluding direct measurement of K_{O_2} by using a simple porphyrin and external axial base. A system enforcing five-coordination about iron (i.e., effectively reducing K_2 to zero) has been developed to permit such measurements (unpublished results).

Carbon monoxide is a biological ligand for Hb and Mb (11–13), and thus the binding of CO to model systems is also of interest. We wish to report a study of the binding of CO to iron(II) "picket fence" porphyrins with hindered and unhindered imidazoles as axial bases. Although these "picket fence" porphyrins displayed the same O_2 affinities as Hb and Mb (1), their CO affinities are significantly greater than those of the hemoproteins. We suggest that steric interactions with CO in the hemoproteins serve to lower the CO affinities, a claim supported both by conclusions reached from the examination of mutant hemoglobins lacking such interactions and by the results from the "picket fence" systems reported here.

MATERIALS AND METHODS

Meso-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinato iron(II) (FeTPivPP) and *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-5-(1-imidazolyl)valeramidophenylporphyrinato iron(II) [$FePiv_3$(5CImP)Por], Fig. 1, were prepared as described (ref. 7; unpublished results). Solvents were distilled and stored under N_2: toluene from Na metal, methanol from $Mg(OMe)_2$, and 1,2-dimethylimidazole (Me_2Im) from Na metal under reduced pressure. All experimental operations requiring an inert atmosphere were carried out in a Vacuum Atmospheres "Dri-Lab" under N_2.

Oxygen-binding equilibria were determined with an apparatus consisting of a cuvette equipped with gas inlet and outlet tubes attached to a pair of calibrated Matheson 600 rotameters which mixed pure N_2 with pure O_2 or with premixed O_2 in N_2 (Liquid Carbonics certified gas mixtures, 5.01% O_2 in N_2 and 0.140% O_2 in N_2). Further details are presented elsewhere (14). Carbon monoxide-binding equilibria were determined with the same apparatus, mixing pure O_2 with premixed CO in N_2 (Airco specialty gas mixture, 0.0478% CO in N_2). Concentrations of metalloporphyrins were $\approx$50 μM in all cases. For FeTPivPP, the concentration of Me_2Im was chosen to provide >98% five-coordinate iron(II) porphyrin, based on the equilibrium constant for formation of the five-coordinate species, as determined by standard spectrophotometric techniques (15).

In all of our work, we have chosen to express equilibrium constants in terms of $P_{1/2}$ (pressure units), the pressure of gaseous ligand at which half of the iron(II) porphyrin is ligated. Other workers (2, 10, 16) however, express equilibrium constants in terms of K (concentration units, mol^{-1} liter). A discussion of the relative advantages of each representation, as well as the assumptions implicit in each, has not appeared.

For comparisons of "intrinsic" behavior independent of solvation effects, the equilibrium of interest is that for isolated molecules in the gas phase:

$$\mathrm{FePB_g} + \mathrm{L_g} \overset{K_g}{\rightleftharpoons} \mathrm{FePBL_g},$$

where L represents, for example, CO or O_2. For this idealized case,

$$K_g = \frac{P_{\mathrm{FePBL}}}{P_{\mathrm{FePB}}\, P_{\mathrm{L}}},$$

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Abbreviations: T, low-affinity; R, high-affinity; Me_2Im, 1,2-dimethylimidazole; *N*-MeIm, 1-methylimidazole; TPivPP, *meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinate; Piv_3(5CImP)Por, *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-5-(1-imidazolyl)varleramidophenylporphyrinate; $P_{1/2}$, pressure at half-saturation.

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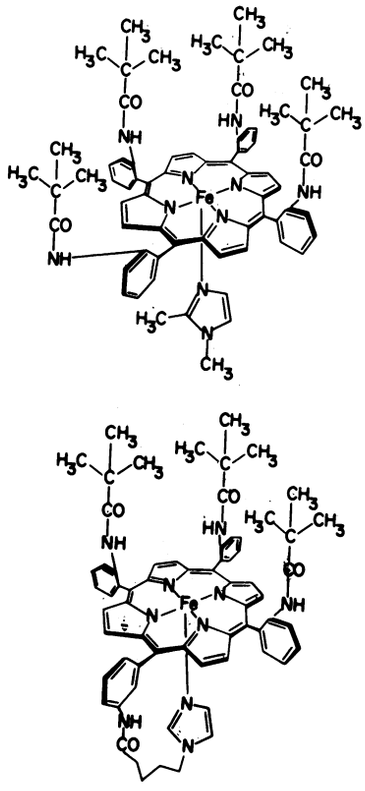

FIG. 1. Five-coordinate, iron(II) "picket fence" porphyrins. (*Upper*) FeTPivPP(Me_2Im); (*Lower*) $FePiv_3$(5ClmP)Por.

where P_X represents the partial pressure of species X. In practice, however, equilibria are studied in solution, and the effects of solvation must be taken into account before comparisons between systems can be made.

We define the equilibrium constant for gaseous versus dissolved species X as

$$X(\text{gas phase}) \overset{K_x^s}{\rightleftharpoons} X(\text{solution})$$

$$K_x^s = \frac{[X]}{P_x}.$$

If we use units of concentration, then

$$K_c = \frac{[FePBL]}{[FePB][L]} = \frac{P_{FePBL}K^s_{FePBL}}{P_{FePB}K^s_{FePB}\,P_LK^s_L}$$
$$= K_g \cdot \frac{K^s_{FePBL}}{K^s_{FePB}K^s_L}.$$

The implicit assumption for $K_c = K_g$ then made in using concentration units is

$$\Delta G^s_{FePB} = \Delta G^s_{FePB} + \Delta G^s_L,$$

where ΔG^s_x represents the free energy of solvation of species X, or

$$K^s_{FePBL} = K^s_{FePB}K^s_L.$$

Thus, given the above assumption, equilibrium constants should be expressed in terms of concentration.

If instead we use units of pressure, then

$$K_p = \frac{[FePBL]}{[FePB]P_L} = \frac{P_{FePBL}K^s_{FePBL}}{P_{FePB}K^s_{FePB}P_L}$$
$$= K_g \cdot \frac{K^s_{FePB}}{K^s_{FePB}}.$$

The implicit assumption for $K_p = K_g$ then made in using pressure units is

$$\Delta G^s_{FePBL} = \Delta G^s_{FePB}$$

or

$$K^s_{FePBL} = K^s_{FePB}.$$

Thus, if the latter assumption is made, then equilibrium constants should be expressed in terms of pressure.

One problem in using concentration units is that the equilibrium constants are then dependent on the solubility of the gaseous ligand L in the solvent of choice. This leads to some confusion in that the Henry's Law constants, describing solubilities of gases, are subject to change and refinement, and any changes in these constants affect the *calculated* values of the equilibrium constants of interest, although, of course, the *actual* values of the equilibrium constants are unaffected by such changes. The use of pressure units, on the other hand, allows direct comparison between various model systems and the proteins, regardless of solvent and Henry's Law constants, and it is primarily for this reason that we choose to express our equilibrium constants in terms of $P_{1/2}$ ($= K_p^{-1}$). The design of the "picket fence" porphyrin, with a protected binding "pocket," is such as to make the solvation energy assumption implied by the use of pressure units a reasonable one. Because the ligand is "buried" in the pocket, the ligated and unligated species may look quite similar to the solvent. The same quite reasonably may be argued for the hemoprotein binding pocket. However, as our results in toluene/methanol show, there is clearly some residual solvent effect with our compound. On the other hand, the ligated and unligated forms of simple "flat" porphyrins may look considerably different to the solvent, and the solvation energy assumption implied by the use of concentration units may be more reasonable in such cases.

Importantly, our conclusions are actually independent of a solvation energy assumption—our model complexes bind CO with higher affinity than do Hb and Mb (*vide infra*), both in terms of $P_{1/2}$ (torr) and in terms of K [mol^{-1} liter, calculated from $P_{1/2}$ by using reported Henry's Law constants (17)]. Recent work by Traylor and coworkers (16) has shown their model compound, in benzene, to bind CO with the same affinity as does Hb in terms of K. In terms of $P_{1/2}$, however, their model binds CO 3–10 times better than does Hb. In this case, one is faced with the difficult task of deciding which, if either, of the solvation energy assumptions discussed above is correct.

Table 1. O_2 affinities of iron porphyrins and hemoproteins

System	Physical state	$P_{1/2}{}^{O_2}$ (25°C), torr	Refs.
Mb (sperm whale)	Aqueous, pH 8.5	0.70	18
Hb (human, R)*	Various†	0.15–1.5‡	19
FeTPivPP(*N*-MeIm)	Solid state	0.49	20
$FePiv_3$(5ClmP) Por	Toluene solution	0.58	1
$FePiv_3$(5ClmP) Por	Toluene/MeOH (1:1)	0.059	This work
Hb (human, T))	Various†	9–160‡	1, 19
FeTPivPP(Me_2-Im)	Toluene solution	38	1

* These are actually the first and fourth intrinsic $P_{1/2}{}^{O_2}$ values.

† Various combinations of 0.1 M NaCl, 0.1 M phosphate, 2 mM inositol hexaphosphate, and 2 mM 2,3-diphosphoglycerate were used.

‡ The ratio of these R and T affinities also varied as a function of conditions, from around 40 to 500.

Table 2. CO affinities of iron porphyrins and hemoproteins

System	Physical state	$P_{1/2}^{CO}$ (20–25°C), torr	M	Refs.
Mb	Aqueous, pH 7–7.5	1.2×10^{-2}–2.8×10^{-2}	25–40	23
Hb (R)	Aqueous, pH 7–7.5	1×10^{-3}–4×10^{-3}*	200–250	24–28
Microperoxidase†	Aqueous, pH 7	4×10^{-4}*‡	—	29
$FeDHMe_2(Im)$§	Benzene solution	2.1×10^{-4}*	—	10
$FePiv_3(5ClImP)Por$	Toluene solution	2.2×10^{-5}¶	26,600	This work
$FePiv_3(5ClImP)Por$	Toluene/MeOH (1:1)	3.0×10^{-6}¶	19,900	This work
Hb (T)	Aqueous, pH 7–7.5	$1 \times 10^{-1} - 2.8 \times 10^{-1}$‡§	32–1600	24, 25
$FeDHMe_2(2\text{-}MeIm)$§	Benzene solution	4.0×10^{-2}	—	10
$FeTPivPP(Me_2Im)$	Toluene solution	8.9×10^{-3}¶	4280	This work
Hb Zurich (β-$His^{63} \rightarrow Arg$)	Aqueous	—	$\gtrsim$500	30

One torr = 1.333×10^2 Pa.
* Calculated from reported K (mol^{-1} liter) by using Henry's Law constants.
† A biological histidine-tailed iron(II) porphyrin.
‡ Calculated from reported kinetic data.
§ $DHMe_2$ = deuteroporphyrinato IX dimethyl ester; Im = imidazole; 2-MeIm = 2-methylimidazole.
¶ Estimated errors ±10%.

RESULTS

Direct spectrophotometric determination of the O_2 affinities of the five-coordinate iron(II) porphyrins, $FeTPivPP(Me_2Im)$ and $FePiv_3(5ClImP)Por$, was reported (7), and the results of these studies are summarized in Table 1. Such measurements were not possible in the case of CO, however; the CO affinities of the five-coordinate iron(II) porphyrins are sufficiently high that extremely low partial pressures of CO are needed, and our apparatus cannot deal with these low pressures in a reliable manner. By examining the competition between O_2 and CO for the five-coordinate porphyrins, reproducible values of the so-called partition coefficient (21), M, the equilibrium constant for the competition:

$$FePB(O_2) + CO \overset{M}{\rightleftharpoons} FePB(CO) + O_2$$

were readily determined. [This technique has been widely used in the study of the CO affinities of various hemoproteins (21).] Sets of spectra were recorded for a range of partial pressures of O_2 and CO. Standard calculations (15), using an over-determined least squares computer program (22), yielded the mole fractions of oxy- and carbon monoxy-complexes present for each set of partial pressures, and these, in turn, yielded the equilibrium constant, M, for the competition. The desired equilibrium constant, $P_{1/2}^{CO}$ [the affinity of a five-coordinate iron(II) porphyrin for CO, expressed as the half-saturation pressure], is easily derived from M, because

$$M = \frac{[FePB(CO)]P_{O_2}}{[FePB(O_2)]P_{CO}} = \frac{K_{CO}}{K_{O_2}} = \frac{P_{1/2}^{O_2}}{P_{1/2}^{CO}}$$

and $P_{1/2}^{O_2}$ has been independently determined for the systems of interest here. The results of these studies, along with comparable data from hemoprotein systems, are summarized in Table 2.

DISCUSSION

The data presented in Table 1 show that the O_2 affinities of the model iron(II) "picket fence" porphyrins compare favorably with the affinities of Mb and Hb, and the implications of this correlation have been fully discussed (1). In contrast, the CO affinities of the model complexes are much greater than those of the hemoproteins, as seen from the data in Table 2.

Single crystal x-ray structural determinations have shown that O_2 is bound in a bent geometry in the "picket fence" porphyrin (Fe-O-O angle $\approx$135°) (31–33), whereas CO is bound linearly (Fe-C-O angle = 180°, normal to the porphyrin plane) (34) (Fig. 2). The structures of several oxy- and carbon monoxyhemoproteins have also been determined. In the proteins, O_2 is bound in a bent geometry (35, 36), as in the model compounds, but CO is not bound linearly; rather, it is tilted (or bent) off axis (37–41) (Fig. 3). Structural analysis has implicated the so-called distal residues, bulky amino acid residues near the binding site [often histidine, but also isoleucine (40) and leucine (41)], as the source of this distortion (39). Although the partial pressure of CO (P_{CO}) in the uncontaminated atmosphere [<0.2 ppm (42)] is too low to affect significantly even the unconstrained iron(II) model compounds, it is well known that a significant quantity of CO is produced *endogenously*; the biological catabolism of Hb and Mb produces 1 mol of CO per heme (11–13). We (43, 44) and others (30, 39, 45, 46) have proposed that the distortion of CO in the hemoproteins serves to lower the affinity of these hemoproteins for CO; without this distortion, the amount of Hb and Mb poisoned by endogenous CO would be much greater than the current estimate of approximately 1% (47). In the absence of steric hindrance, the endogenous P_{CO} would produce greater than 50% HbCO and MbCO (based on a value of M of approximately 20,000), at which point the functioning of these proteins would be critically impaired. The model compounds clearly present no steric hindrance to bound CO, and, as the data of Table 2 show, the CO affinities of the models *are* significantly greater than those of the hemoproteins.

Infrared spectroscopy has also been used to examine the binding of CO to hemoproteins and model compounds (26, 45); the pertinent data are presented in Table 3. Possibly, distortion

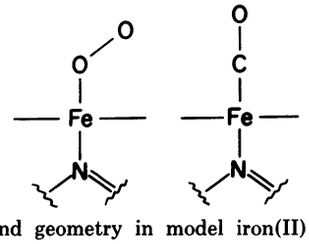

FIG. 2. Ligand geometry in model iron(II) porphyrin complexes.

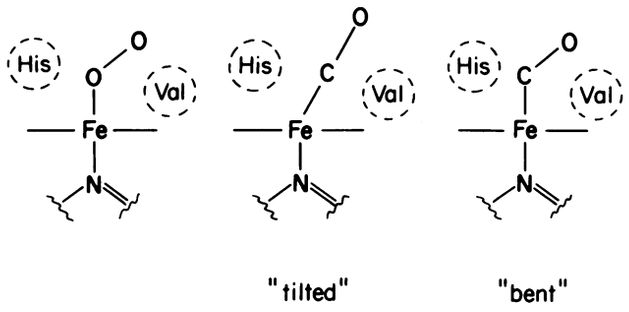

FIG. 3. Ligand geometry in hemoprotein complexes, showing the "distal" residues, β-His^{63} and β-Val^{67}.

of CO is correlated with a decrease in ν_{CO}.† Though electronic effects on ν_{CO} are also potentially significant (50, 51), distortion of CO is observed in hemoglobins lacking distal groups capable of interacting electronically with CO (40, 41), suggesting that steric interaction is the common denominator in CO affinity reduction among the various hemoproteins.

Further support of the postulated function of steric hindrance to CO binding in hemoproteins comes from an examination of mutant hemoglobins in which the distal residues of the β chains are altered, notably Hb Zurich (β-His^{63} → Arg) and HbM_{Emory} (= $HbM_{Saskatoon}$ = $HbM_{Chicago}$ = HbM_{Kurume} = HbM_{Radom}) (β-His^{63} → Tyr) (52). These mutants have ν_{CO} for the β chains approaching that of unconstrained porphyrins (49) and of denatured Hb (48): ν_{CO} (Hb Zurich) = 1951 cm^{-1} (α chains), 1958 cm^{-1} (β chains); ν_{CO} (HbM_{Emery}) = 1950 cm^{-1} (α chains), 1970 cm^{-1} (β chains). This has been taken as an indication that the binding site in these mutants is less constrained than in "normal" Hb (HbA) (53). The structure of Hb Zurich has been determined (50) and confirms the openness of the binding pocket. Most significantly, both the "on" rate of recombination of protein with CO (46) and the actual value of the partition coefficient M (47) have been measured for Hb Zurich. In both studies, the Hb Zurich showed marked heterogeneity, due to the difference in reactivity between the normal α chains and the mutant β chains. Whereas the α chains displays "on" rates of recombination and M values quite similar to HbA, the β chains differed noticeably; the "on" rate was significantly greater, and the M value was at least twice that for HbA (see Table 2). Although the M value for HbM_{Emory} has not been determined, it has been found that the β chains of this mutant Hb recombine with CO 20 times faster than do the α chains (54). It appears then, that mutant hemoglobins lacking distal residues that can sterically force CO off axis have higher CO "on" rates and higher affinities than does HbA, supporting the proposed role of these residues in lowering the CO affinities of normal hemoproteins. [In contrast, these mutant hemoglobins bind O_2 with nearly the same affinity as HbA (55, 56), suggesting that steric interactions are not important in the binding of O_2 to hemoproteins, in support of earlier conclusions (43, 44).]

The O_2 affinity of FeTPivPP(Me_2Im) was found (1) to parallel that of the T (low-affinity) state of Hb (see Table 1), and was approximately 75 times lower than that of the unconstrained complex, $FePiv_3$(5ClmP)Por. This decrease in affinity was ascribed to a severe steric interaction between the 2-methyl group of the imidazole and the porphyrin ring, developing as the iron moves toward the porphyrin plane upon becoming six-coordinate. [X-ray crystallography has shown the dramatic effects of this steric interaction (57).] The CO affinity of FeTPivPP(Me_2Im) was also determined (Table 2) and was found to be approximately 400 times lower than that of the unconstrained complex. Various explanations of the larger magnitude of this change relative to that for O_2 are plausible. Simple steric arguments, however, require knowledge of the structure of FeTPivPP(Me_2Im)(CO), and such a structure (or a similar one) has not yet been determined. A similar effect of a sterically hindered axial base upon CO binding has been observed by Rougée and Brault (10).

The effect of solvent polarity in CO binding was also investigated. In a more polar medium [toluene/methanol (1:1)], the CO affinity of $FePiv_3$(5ClmP)Por was nearly an order of magnitude larger than in a less polar medium (toluene). The difference in affinity in the different solvents can be ascribed to either a change in solvation in the binding pocket or to a stabilization of charge separation; the present results do not allow us to distinguish these. Of interest is the value of M, which remains relatively constant on going from toluene to toluene/methanol (1:1); perhaps M is the most significant parameter with which to compare model compounds to the hemoproteins, because it seems to be only slightly dependent on the solvent environment of the porphyrin, as would be expected if the ligated forms are solvated comparably.

Traylor has recently reported (16) on the insensitivity of the CO affinities of his "flat" porphyrins to solvent polarity. A marked solvent dependence of the O_2 affinities of such porphyrins was observed (58). Taken together, these observations demonstrate the solvent dependence of the M values for these porphyrins. This suggests that solvation differences in ligated and unligated "flat" porphyrins are significant, whereas solvation effects appear to have been largely eliminated in the case of the "picket fence" porphyrins. Thus, the "picket fence" porphyrins, with their protected binding site, may be better models for the hemoproteins.

† This is a rather surprising result and is far from understood. Model compounds forcing bent or tilted CO would clearly be beneficial.

Table 3. Infrared stretching frequencies for CO adducts of iron porphyrins and hemoproteins

System	Physical state	ν_{CO}, cm^{-1}	Refs.
MbCO*	Aqueous solution	1945	26
HbCO*	Aqueous solution	1951	26
Denatured HbCO	KBr pellet	1970	48
HbCO Zurich (β-His^{63} → Arg)	Aqueous solution	1951, 1958	49
$HbCOM_{Emory}$ (β-His^{63} → Tyr)	Aqueous solution	1950, 1970	49
FeP(*N*-MeIm)CO†	Benzene solution	1970	48
FeTPivPP(*N*-MeIm)CO	Benzene, CH_2Cl_2, or $CHCl_3$ solution; Nujol mull	1969	43

* These values are typical for various Mbs and Hbs.
† P = protoporphyrinato diethyl ester.

CONCLUSIONS

Though iron(II) "picket fence" porphyrin complexes have been found to bind O_2 with the same affinity as do Hb and Mb, the same model compounds bind CO with significantly higher affinity than do normal hemoproteins. Apparently, steric bulk near the binding site in hemoproteins distorts the FeCO geometry and lowers the CO affinity of these proteins relative to the unconstrained models, thus preventing poisoning from CO produced endogenously from heme catabolism. Mutant hemoglobins lacking steric protection of the binding pocket display CO affinities intermediate between normal hemoproteins and the model compounds and, as such, support the postulated function of the distal histidine in lowering CO affinity.

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